

NITROGEN FIXATION BY EPIPHYLLS IN A TROPICAL RAINFOREST¹

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ABSTRACT

Bluegreen algae (Cyanobacteria) growing on the leaf surfaces of understory plants in a tropical rainforest can fix atmospheric nitrogen. Rates of fixation are strongly influenced by the presence of glucose and mineral nutrients leached from the host leaf, by light intensity as it relates to the photosynthetic rates of the algae, and by desiccation especially as it is influenced by the co-occurrence of epiphyllous bryophytes. A significant portion of this newly fixed nitrogen is transferred to the host leaf and may account for 10–25% of the total nitrogen in a leaf.

Although the major source of nitrogen for plant growth and reproduction is from decomposition of organic matter, most ecosystems can gain significant quantities from precipitation and biological fixation of atmospheric nitrogen. In fact, Burns & Hardy (1975) estimated that biological fixation alone may account for 43% of the nitrogen transferred worldwide. Subba Rao (1977) suggested that the rates in tropical areas may be even higher and thus fixation could have a major role in the tropics where available nitrogen can be limited.

In this paper, I will discuss one aspect of nitrogen fixation in a tropical ecosystem—that of fixation by epiphylls in a rainforest understory. Although epiphyll fixation can account for up to 25% of the nitrogen in a host leaf (Bentley & Carpenter, 1980), rates of fixation are quite variable in both time and space. Here I document some of the environmental factors that influence fixation rates.

What are epiphylls? Epiphylls are epiphytes that are restricted to the surfaces of leaves. In tropical rainforests, most visible epiphylls are leafy liverworts of the family Lejeuneaceae but may also include mosses, lichens and, on some occasions, seedlings of other epiphytes such as bromeliads and orchids. In this study, we focused on the microorganisms, especially the bluegreen algae (Cyanobacteria) growing in association with the visible forms (Fig. 1).

The epiphyll community is best developed in regions of high rainfall and low evaporation and is most diverse in tropical rainforests (Richards,

1964). Because they grow on photosynthetic surfaces, epiphylls are often considered to be detrimental to the host plant by interfering with light penetration to the leaf (Richards, 1964). Indeed, some epiphylls may actually be semiparasitic. For example, the rhizoids of *Radula flaccida*, an epiphyllous liverwort, penetrate the cuticle and absorb nutrients from their host leaf (Berrie & Eze, 1975).

Other workers feel that the presence of epiphylls increases the time that leaves remain wet and thus contributes to the growth of potentially pathogenic bacteria and fungi (Gregory, 1971; Stahl, 1891). Long-term wetting may also reduce the rates of transpiration and subsequent mineral uptake by the roots (Stahl, 1893; McLean, 1919). As early as 1891, Jungner suggested that rainforest plants may have adaptations such as leaf “drip tips” to increase the rates of drainage off the leaf and reduce the rate of colonization by epiphylls. Subsequent work has failed to either deny or confirm the adaptive role of these characters (Stahl, 1893; Shreve, 1914; Seybold, 1957; and pers. obs.).

Nitrogen fixation by epiphylls. Some species of epiphyllous microorganisms are known to fix nitrogen (Bentley & Carpenter, 1980, 1984; Ruinen, 1975). During our study at the La Selva Biological Station in Costa Rica, we found that fixation rates are extremely variable, both within and among species (Fig. 2). Interestingly, in contrast to Ruinen’s data where fixation was by free-living bacteria (primarily *Beijerinckia*), fixation in the La Selva understory was most commonly

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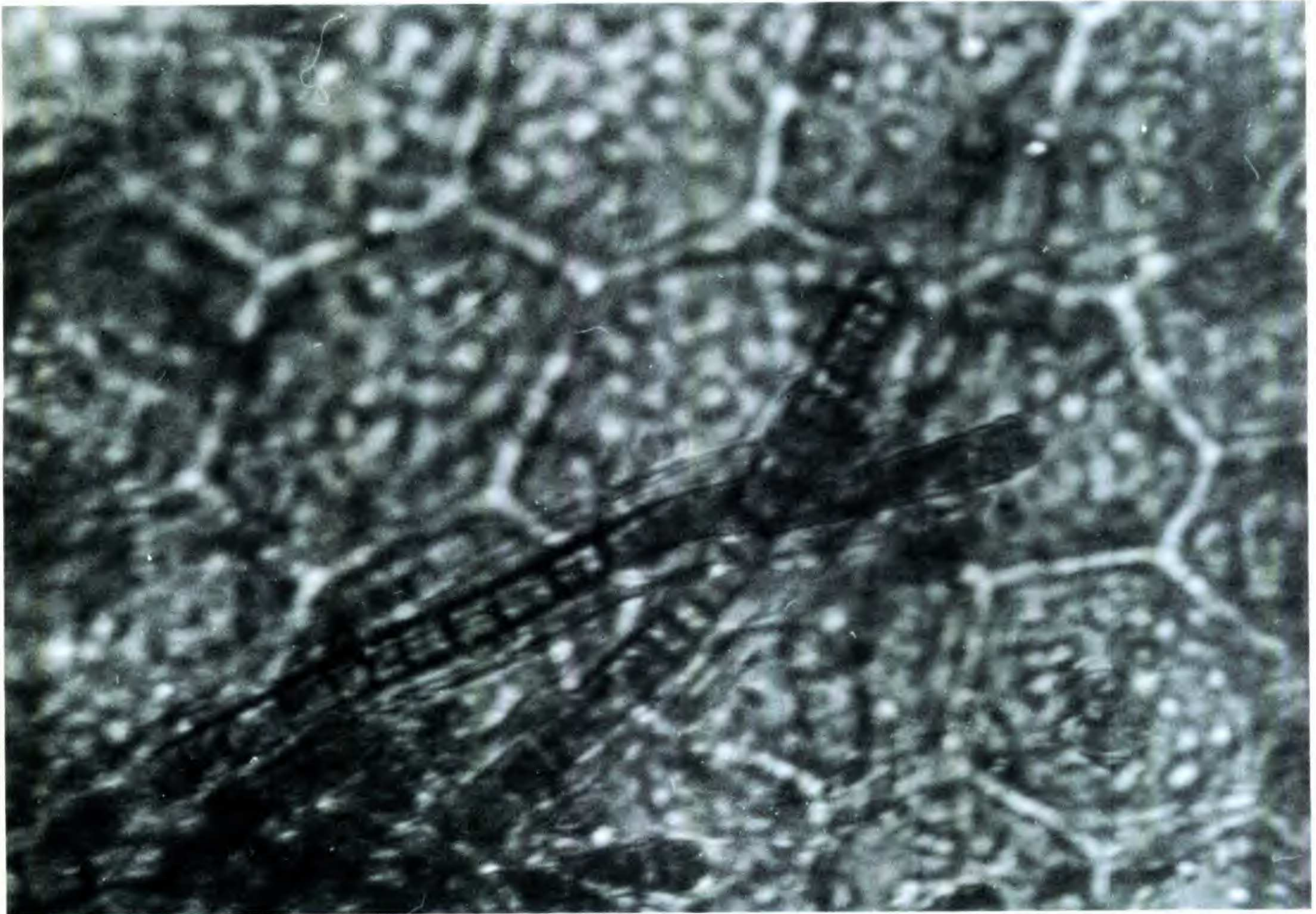


FIGURE 1. Photomicrograph of epiphylls. The strand of the nitrogen-fixing bluegreen alga is across the lower third of the photograph.

associated with the presence of bluegreen algae in the genera *Scytonema*, *Stigonema*, and *Hapalosiphon*. High fixation rates were invariably associated with a dense cover of bryophytes, suggesting that the bryophytes provide a good substrate for the nitrogen-fixing microorganisms.

Most of our work on fixation was done using the acetylene reduction method for determining nitrogenase activity (Bentley & Carpenter, 1980; Prestwich & Bentley, 1981; Burris, 1972). While this is an extremely easy field assay for estimating fixation, it is not a direct measure of *nitrogen* fixation, and cannot be used to answer the most critical question in our study: does the newly fixed nitrogen get into the host leaf? By using ^{15}N as a tracer, we were able to document that indeed this is the case (Bentley & Carpenter, 1984). There we showed that nitrogen fixation by epiphylls could account for up to 25% of the nitrogen in a host leaf.

In other studies with bluegreen algae, Stewart (1963) and Jones & Stewart (1969) found that the extracellular nitrogenous products are primarily amino acids or peptides, but less complex compounds, including ammonium nitrite and

nitrate, can be present. The pathway for movement of the new nitrogen is not through the stomata, as might be assumed, but rather to the epidermal cells via threadlike ectodesmata penetrating through the cell wall to the cuticle (Franke, 1970). Because of the specific morphology of the ectodesmata, Franke felt that the movement of soluble materials into and out of the leaf is a normal process, closely correlated with foliar absorption of "foreign" substances such as fertilizers and pesticides.

Environmental factors affecting fixation. The rates of fixation and transfer that we measured were made under ideal conditions for the activities of microorganisms. Although the high temperatures and almost constant moisture in a tropical rainforest can permit high fixation rates and concomitant release of nitrogenous products, we have also observed extremely high variance both among and within species at the La Selva Station (Fig. 2). Thus, it becomes important to ask what environmental factors influence fixation rates by epiphylls. Basically, the answer lies in three factors: time, moisture, and nutrients.

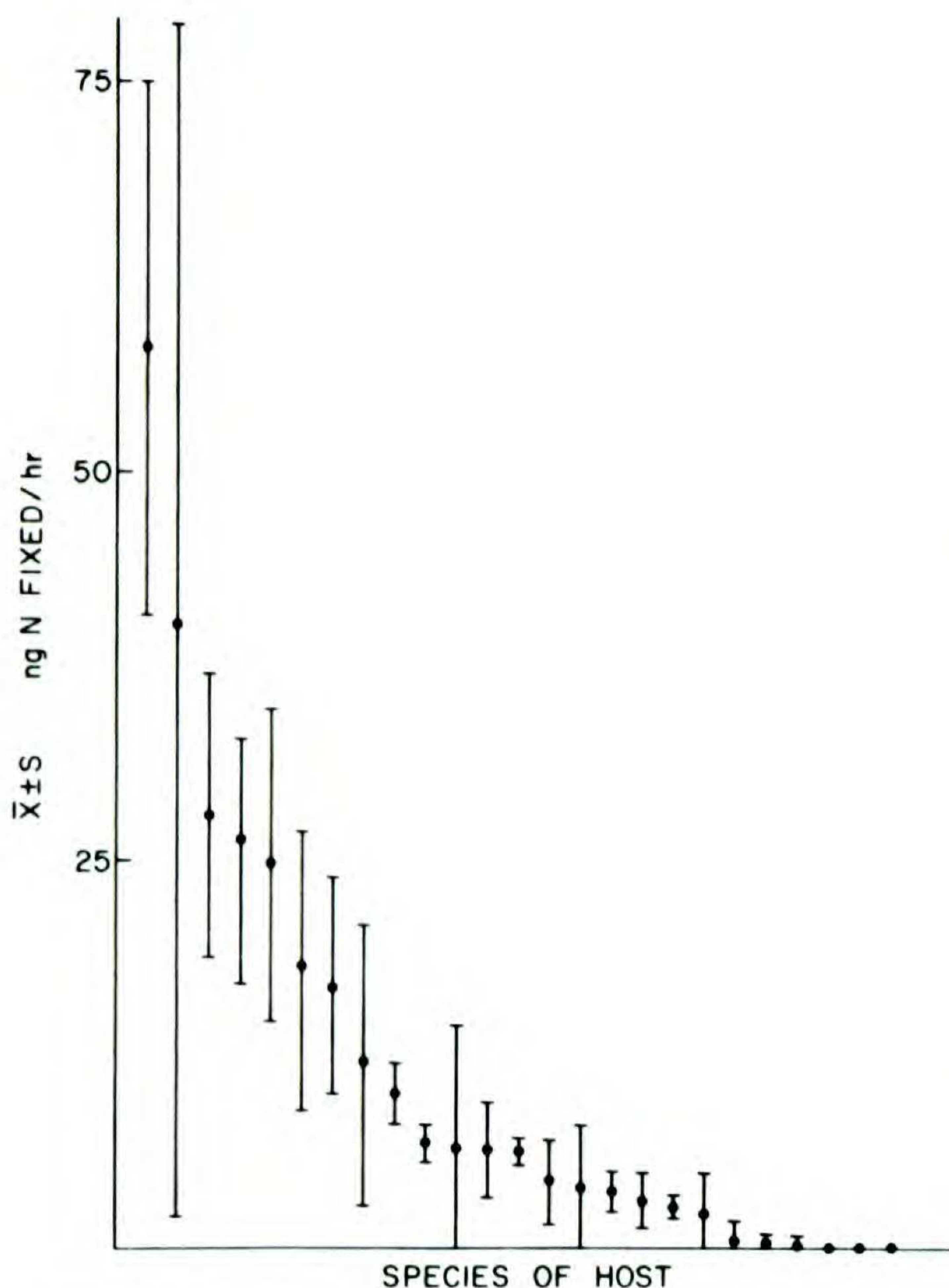


FIGURE 2. Nitrogen fixation rates by epiphylls on the surfaces of various host leaves. Means (O) and variance (error bars) are for five samples from each host. The host species, from highest to lowest fixation rates, are: *Ocotea atirrhensis*, *Proteum pittieri*, *Chamaedorea* sp., *Ficus* sp., *Swartzea* sp., *Miconia* sp., *Piper* sp. 1, *Piper* sp. 2, *Polypodium* sp., *Theobroma cacao*, *Inga* sp., *Costus* sp., *Syngonium* sp., *Asterogyne* sp., and *Geonoma* sp.

Time. Colonization by epiphylls is a time phenomenon. Young leaves are epiphyll-free simply because the epiphylls have not become established. Long-lived leaves have a much greater probability of having a well-established epiphyll community. To document the role of leaf longevity on fixation, we measured fixation on leaves which had been previously marked for up to 24 months (Bentley, 1979). Since we knew the average survivorship of leaves on a plant, we could then test for the correlation between the age of a leaf and the rate of fixation. As can be seen in Figure 3, fixation rates are significantly correlated with leaf longevity. At the same time, we measured fixation by leaves with differing levels of epiphyll biomass. Again, there is a significant correlation (Fig. 4).

Moisture. Interestingly, in the experiments described above, the rates of fixation were correlated with the biomass of the bryophytes, even

though bryophytes per se do not fix nitrogen. On the other hand, bryophytes are correlated with and can influence the levels of moisture on the leaf surface (Richards, 1964). Since moisture can affect fixation rates in other systems (Balandreau et al., 1974; Horne, 1972; Fogg et al., 1973), we set up an experiment to test the effects of desiccation on fixation by epiphylls. In this case, individual pinnae of *Welfia georgii* were moistened with water 12, 4, 2, or 0 hours before incubation. As can be seen in Figure 5, desiccation has a very dramatic effect on fixation, yet the recovery is quite rapid and is almost that of the continuously moist control within four hours.

Although moisture conditions in a tropical rainforest are relatively constant compared with most other ecosystems, moisture conditions in the understory do vary, both seasonally and diurnally (Longman & Jenik, 1974; Ruinen, 1961). For example, water may continue to drip down through the forest canopy long after a rain has ceased, but the leaves of the upper canopy will dry within minutes. Seasonal variation can range from an increased frequency of dry periods in a day to days or weeks without any appreciable rainfall (Schnell, 1971). Thus, under field conditions, moisture can have significant effects. We documented this in another experiment, shown in Figure 6. In this case we found that fixation rates on rainy days were considerably higher than on dry days.

Although it is tempting to suggest that the bluegreens and the bryophytes have some close symbiotic relationship, the role of bryophytes is probably no more than to maintain appropriate moisture conditions on the surface of the leaf. Epiphyll-laden leaves dry more slowly, not only because capillary action holds water but also because bryophytes can draw water directly from the interior of the leaf (Berrie & Eze, 1975). In addition, the epiphyll-laden portion of a leaf surface will become wetter faster, again because of capillary action drawing water from surrounding areas. High fixation rates by algae are correlated with the presence of bryophytes simply because the moisture conditions surrounding epiphyllous bryophytes are appropriate for the growth and activity of microorganisms.

Energy sources. Nitrogen fixation consumes energy—free-living microorganisms may require 200 g of glucose for every gram of nitrogen fixed (Mulder, 1975). Unlike other microorganisms, bluegreen can use carbohydrates from photosyn-

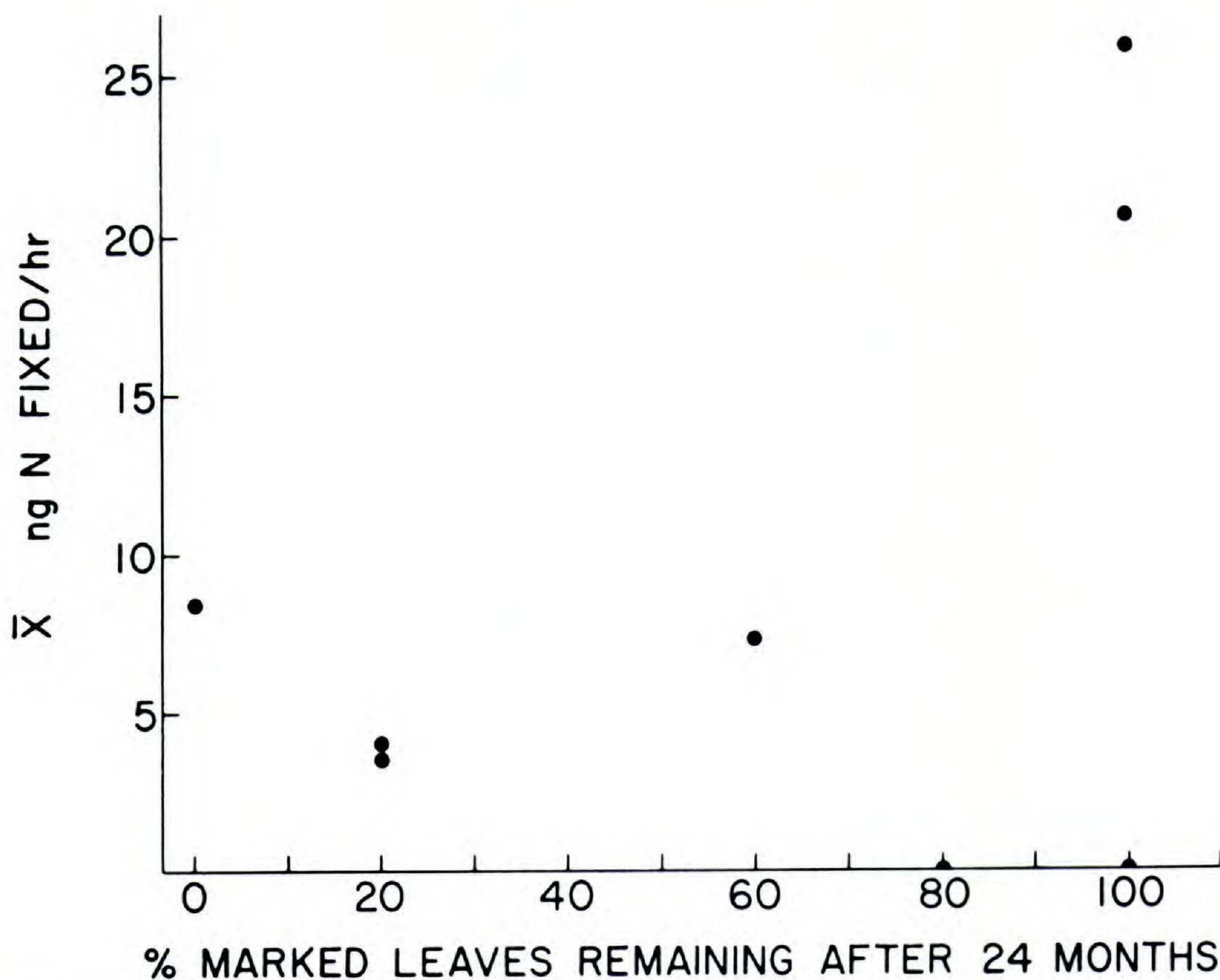


FIGURE 3. Nitrogen fixation rates by epiphylls on plants with different leaf longevities. Highest rates are on *Ocotea atirrhensis* in which all leaves live longer than 24 months. Lowest rates were on leaves of *Piper* (80% last less than two years). The rank correlation of fixation with longevity is significant ($r = 0.95$, $P < 0.01$).

thesis as well as exogenous carbon to supply the nitrogen fixation process. In addition, bluegreens may be able to use intermediate products of photosynthesis to provide electrons for nitrogen fixation (Mulder, 1975).

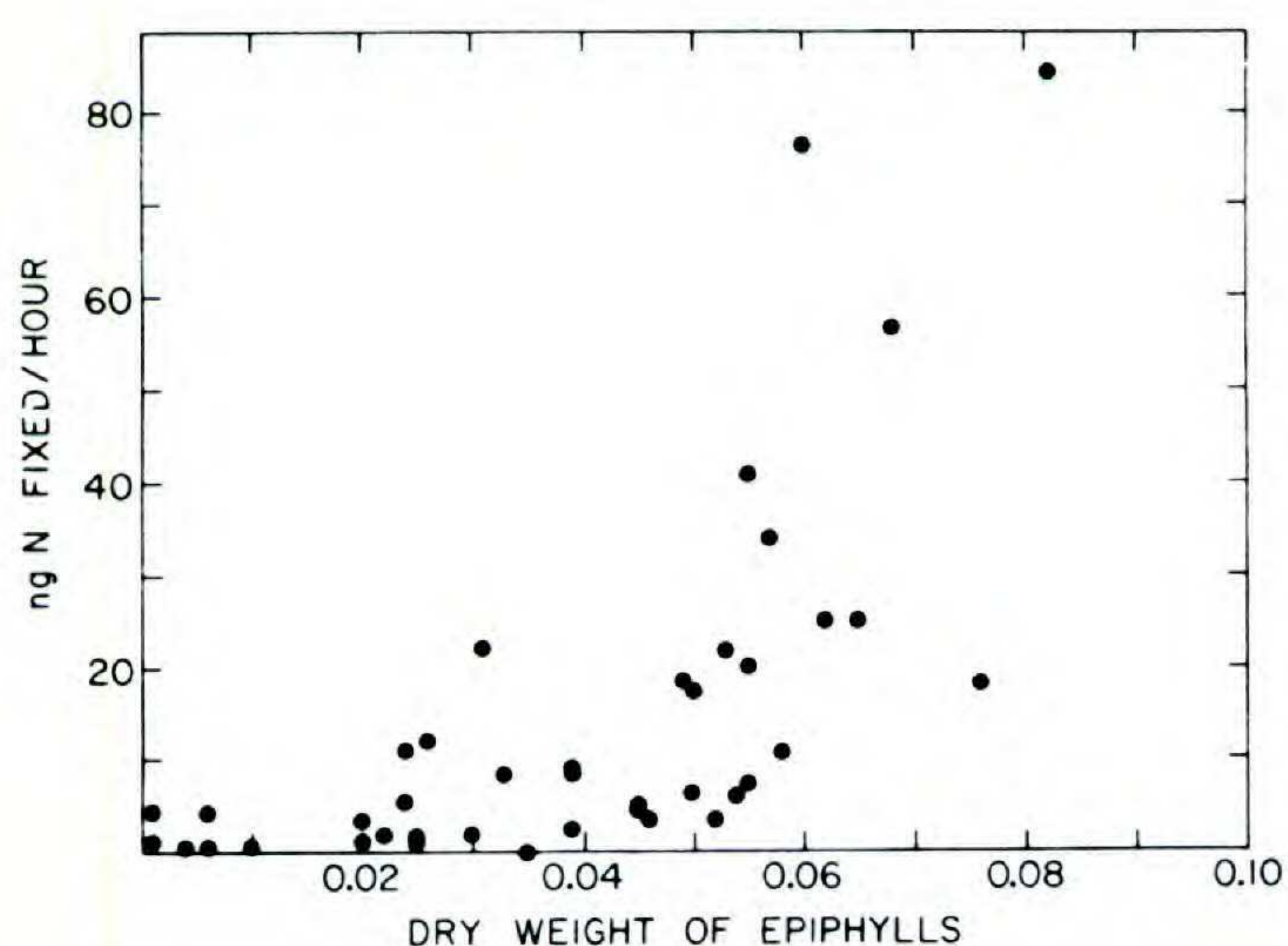


FIGURE 4. The relationship of epiphyll biomass and fixation rates. Fixation rates were measured on intact 2 cm × 5 cm leaf samples. After incubation, the epiphylls were removed by scraping and dried at 80°C for 24 hours. The epiphyll biomass includes both bryophytes and bluegreens. The correlation between fixation and biomass is significant at the $P < 0.05$ level ($r = 0.39$).

In the field, nitrogen fixation by bluegreen algae is usually light dependent (Henricksson & Simu, 1971; Horne & Viner, 1971; Stewart, 1973; and see Fig. 6) and has the same relationship to light as photosynthesis—decreasing with decreasing light intensity. However, low rates of fixation can occur in the dark (Forman, 1975; Dugdale & Dugdale, 1962), especially if the organisms are grown on substrates containing glucose, fructose, or sucrose (Fay, 1965). Thus, many workers feel that high rates of fixation under natural conditions are a function of both photo-mediated internal processes as well as various forms of exogenous carbohydrates.

Exogenous carbohydrates may come from a wide variety of sources. The major source for epiphylls is in leachate from the host leaf (Tukey & Morgan, 1962; Tukey, 1971). Since the amount of carbohydrate present in leachate is also a function of the photosynthetic rate of the host leaf (Tukey et al., 1957), the amount of exogenous carbohydrate available to the epiphylls will also be correlated with light intensity in the field.

To document the effects of light on the epiphyll system, we set up two series of experiments: the first to establish that levels of light commonly found in the rainforest understory could influ-

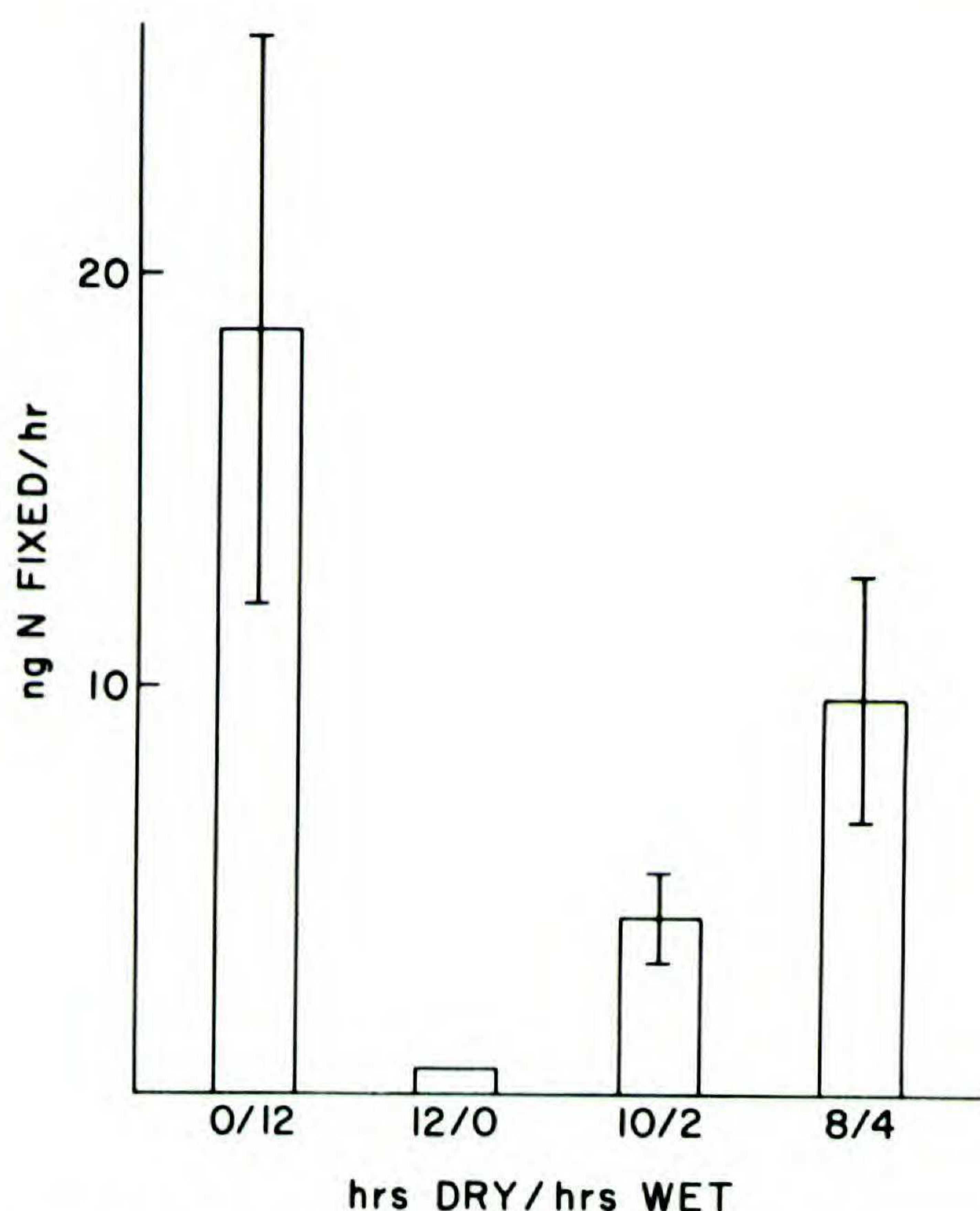


FIGURE 5. The effects of desiccation on nitrogen fixation by epiphylls. Leaflets from the same pinna of *Welfia georgii* were either kept constantly wet (0 hr. dry; 12 hr. wet), or were subjected to varying lengths of drying (12 hr. dry, 0 hr. wet; 10 hr. dry, 2 hr. wet; or 8 hr. dry, 4 hr. wet).

ence fixation rates, and the second to differentiate between direct effects of light and effects of exogenous glucose on fixation rates by the blue-greens. In the first set of experiments, samples were incubated at three levels of light set to mimic those found in the forest: over epiphyll-laden leaves ($12 \mu\text{Einsteins m}^{-2}$), in a light gap ($68 \mu\text{Einsteins m}^{-2}$), and in the dark. As can be seen in Figure 7, fixation was highest at the highest light intensity and was still significant even at the usual light intensity for most epiphylls.

In the second set of experiments, we measured nitrogen fixation by leaf samples that had been dipped in a 0.1% glucose solution, a concentration chosen to mimic concentrations we measured in leaf leachate (Tukey et al., 1957). Again, we incubated the samples at two light intensities. In this case, we were able to separate out the effects of light from the effects of exogenous glucose. Note in Figure 8 that fixation is highest in those samples which were both dipped in the glucose solution and incubated in the light. The lowest rates were for the control samples dipped in water and incubated in the dark. Note, however, that fixation by the samples dipped in the sugar solution but incubated in the dark contin-

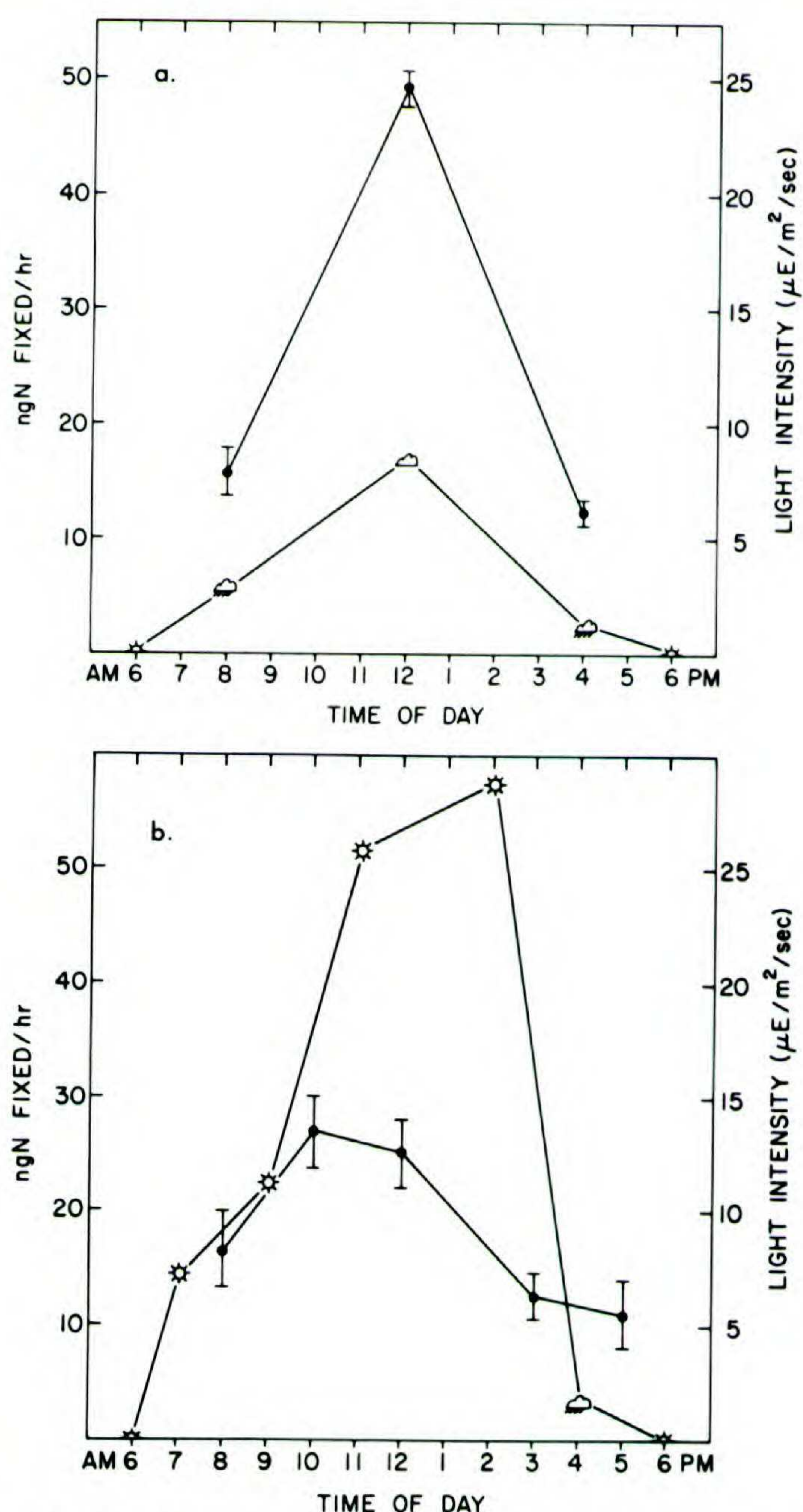


FIGURE 6. The effects of light intensity on nitrogen fixation rates by epiphylls. —a. Fixation rates measured at three times during day 1. Natural light intensities changed with time of day and degree of cloudiness. —b. Fixation rates measured at five times during day 2. Natural light intensities were abnormally high because of the lack of cloud cover.

ued to fix nitrogen at a reasonable rate for at least eight hours. These results document that fixation by epiphylls is influenced by both light directly and by the presence of exogenous glucose. And perhaps as important, they document that fixation can continue at night if exogenous glucose is present on the leaf surface.

Mineral nutrients. Growth of nitrogen-fixing microorganisms may also be limited by availability of mineral nutrients—most commonly potassium, phosphorus, calcium, molybdenum, and iron (Mulder, 1975; Fogg, 1973; Mague, 1977). Deficiencies in any of these can have an

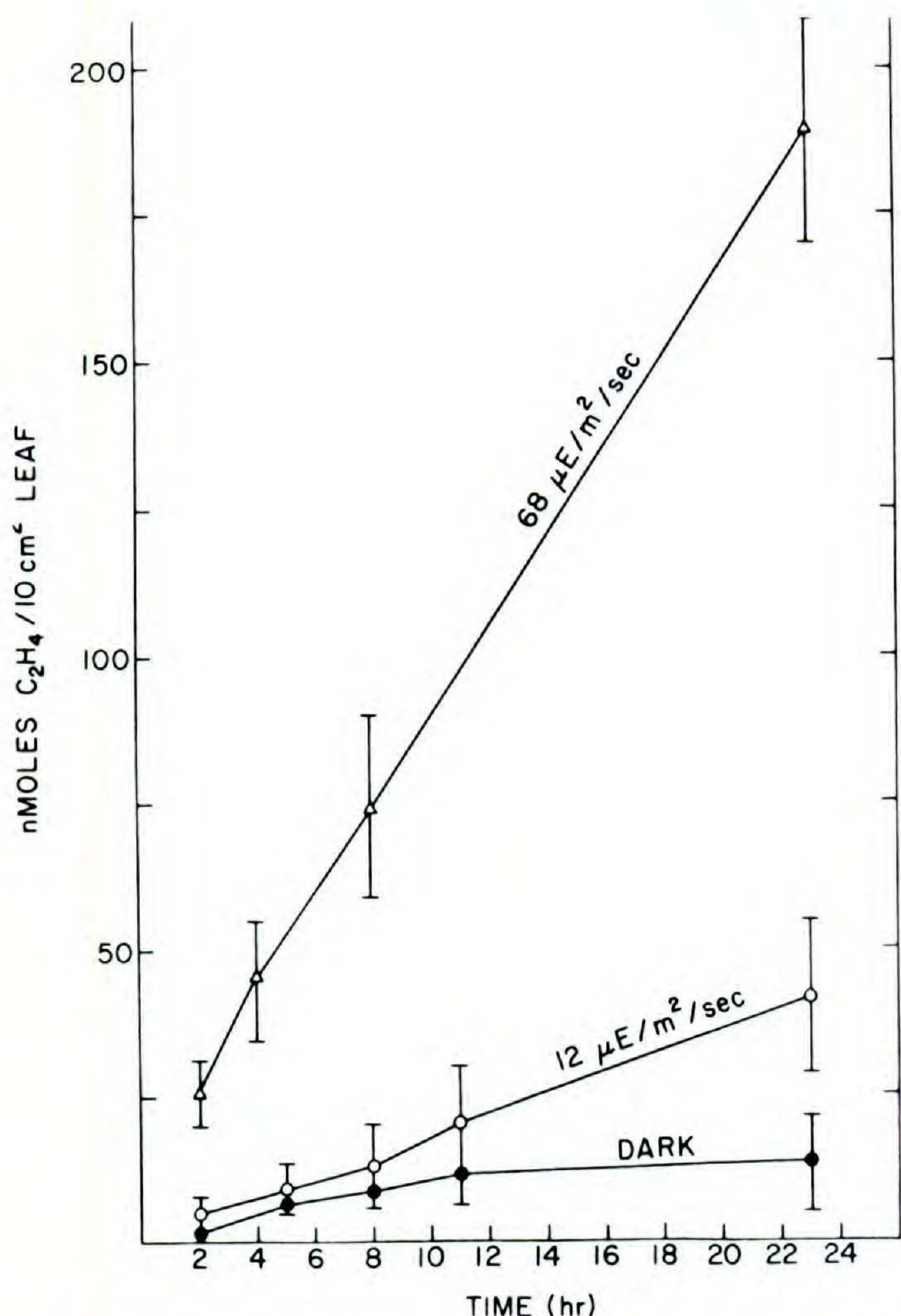


FIGURE 7. Experimental variation of light intensities. Fixation rates are given in terms of nM ethylene to illustrate continuing fixation throughout the 24 hours of the experiment. Ethylene concentrations of samples incubated in the dark do not change after 12 hours.

indirect effect on nitrogen fixation because each is involved in general metabolic activities. In addition, Mo and Fe are components in the nitrogenase molecule. Deficiencies in either of these reduces rates of enzyme synthesis and thus directly affect fixation (Burns & Hardy, 1975).

Exogenous combined nitrogen, most notably NH_4^+ , will also inhibit nitrogen fixation (Stewart, 1973; Burns & Hardy, 1975; Fogg, 1973). Again, the effect is the result of reduced nitrogenase synthesis. Interestingly, the effects of combined nitrogen may explain why fixation rates are low in nongrowing cells: as growth slows down, nitrogenous products accumulate in the cell, which in turn impacts on enzyme synthesis. Thus, deficiencies in *any* substance required for growth may reduce nitrogenase activity.

As with exogenous carbohydrates, the main source of mineral nutrients is through decomposition of organic litter on the forest floor, although some may be available in leachate, stem-flow, or throughfall water (Long et al., 1956). The

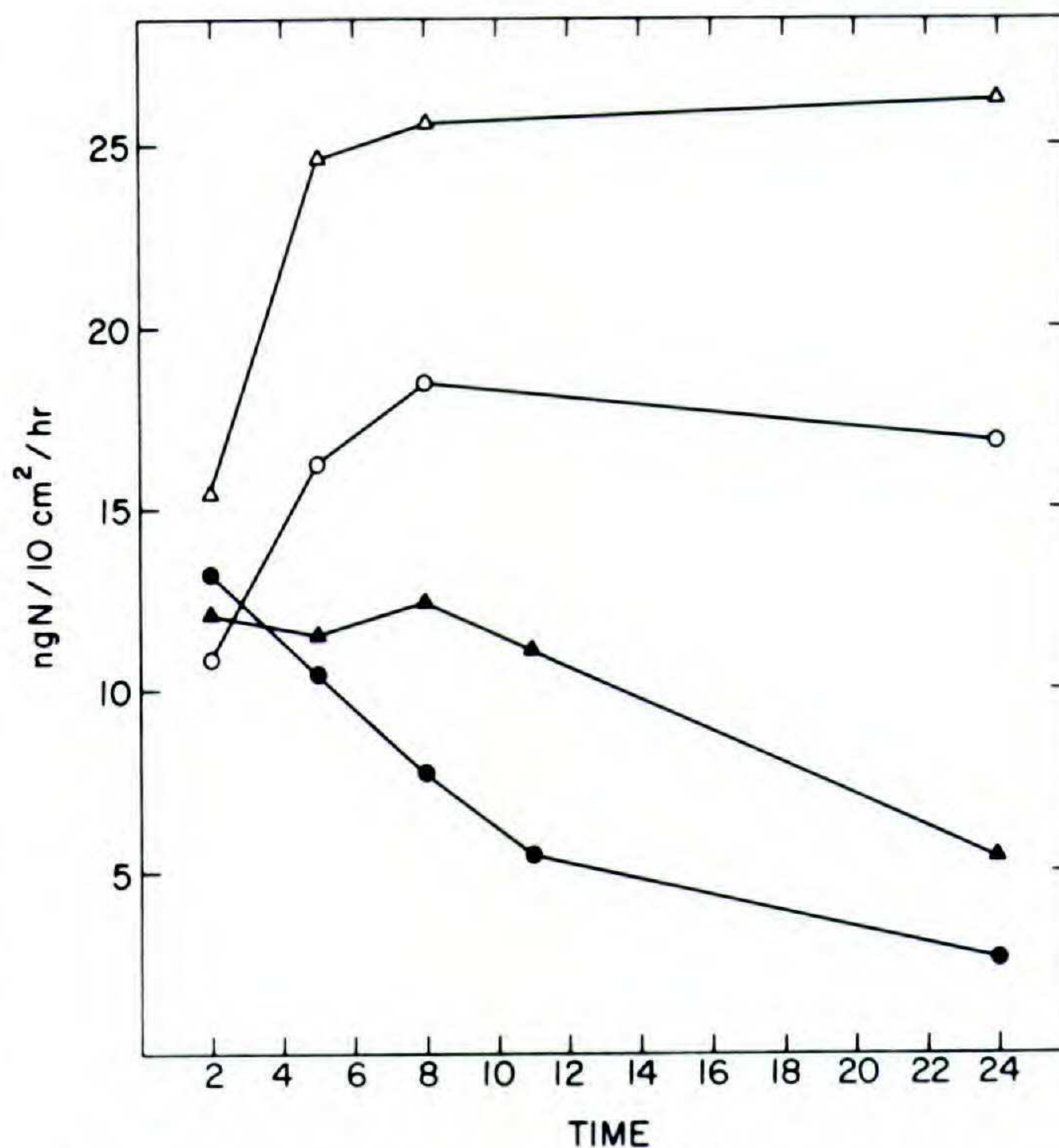


FIGURE 8. The influence of light and exogenous glucose on nitrogen fixation by epiphylls. Samples were incubated in the dark (solid symbols) or light (open symbols) and were either dipped in a 0.1% glucose solution (triangles) or deionized water (circles). The light treatments are significantly different after 4 hours ($P < 0.05$) and all treatments are significantly different after 8 hours ($P < 0.01$).

latter, in fact, might be quite important in rain-forest systems: often a large proportion of decomposition occurs before the dead materials reach the forest floor (Duvigneaud & Denaeyer-de Smet, 1970; UNESCO, 1978). Thus, stem-flow and throughfall water can contain significant quantities of nutrient ions derived from "pre-fall" decomposition as well as that leached from living tissue.

To document the effects of mineral nutrients under field conditions, we performed two types of experiments. The first was to simply measure the nutrients present in rainfall, throughfall, and water flowing off the surfaces of epiphyll-laden leaves, and the second was to determine if nutrients in surface waters could affect fixation rates. We chose to measure phosphate and ammonium simply because these two ions have the most direct effects on fixation. As can be seen in Figure 9, both phosphate and ammonium are present in the environment. Interestingly, the water flowing off epiphyll-laden leaves actually has less nitrogen than the rainfall collected in the open. This suggests that the uptake mechanisms on the leaf surface are extremely efficient. As expected, the concentrations of these two nutrients were

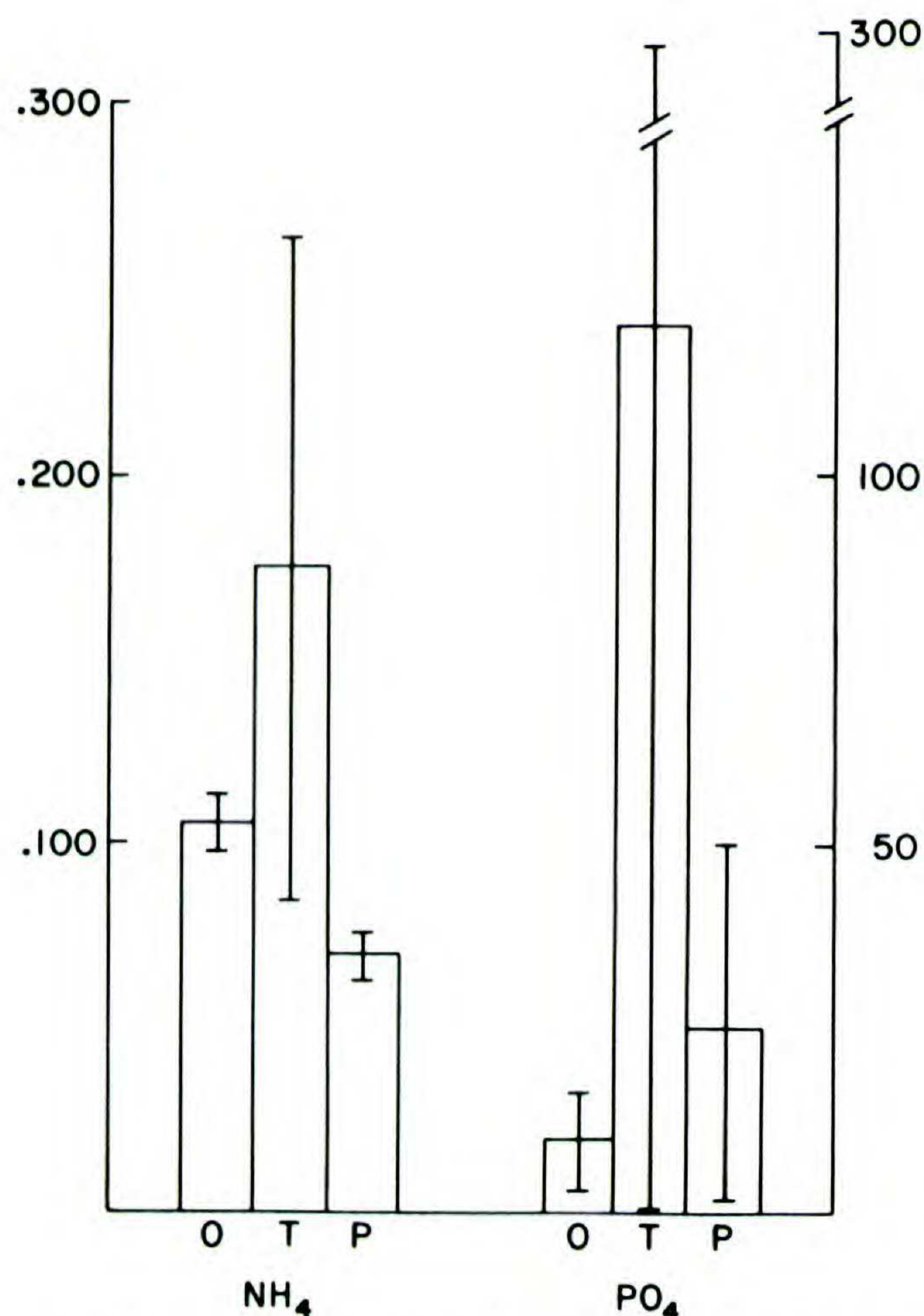


FIGURE 9. Concentrations of ammonium ($\mu\text{g}/\text{ml}$, left axis) and phosphate ($\mu\text{g}/\text{liter}$, right axis) in open-collected (O) rainwater, throughfall (T), and from epiphyll-laden leaflets of *Welfia georgii* pinnae (P). Note the extremely high variance (error bars) in the throughfall values.

most variable in water collected as throughfall. Since these nutrients can affect fixation rates (Fig. 10), the concentrations of nutrients in the water washing over the leaf surface can be yet another component adding to the variances of fixation observed in the field.

Summary and conclusion. During the course of this study, we have documented that epiphyllous microorganisms can fix significant quantities of atmospheric nitrogen. This nitrogen can be absorbed by the host leaf and thereby contributes to the nitrogen economy of the host. However, fixation rates are extremely variable and are strongly influenced by environmental factors including substrate (leaf) longevity, light and concomitant desiccation, both organic and inorganic nutrients, and by the co-occurrence of bryophytes. Thus, while fixation by epiphylls can account for up to 25% of the nitrogen present in a host leaf, the contribution of new nitrogen by epiphylls to an ecosystem is probably fairly small. Nevertheless, the very patchiness of the process

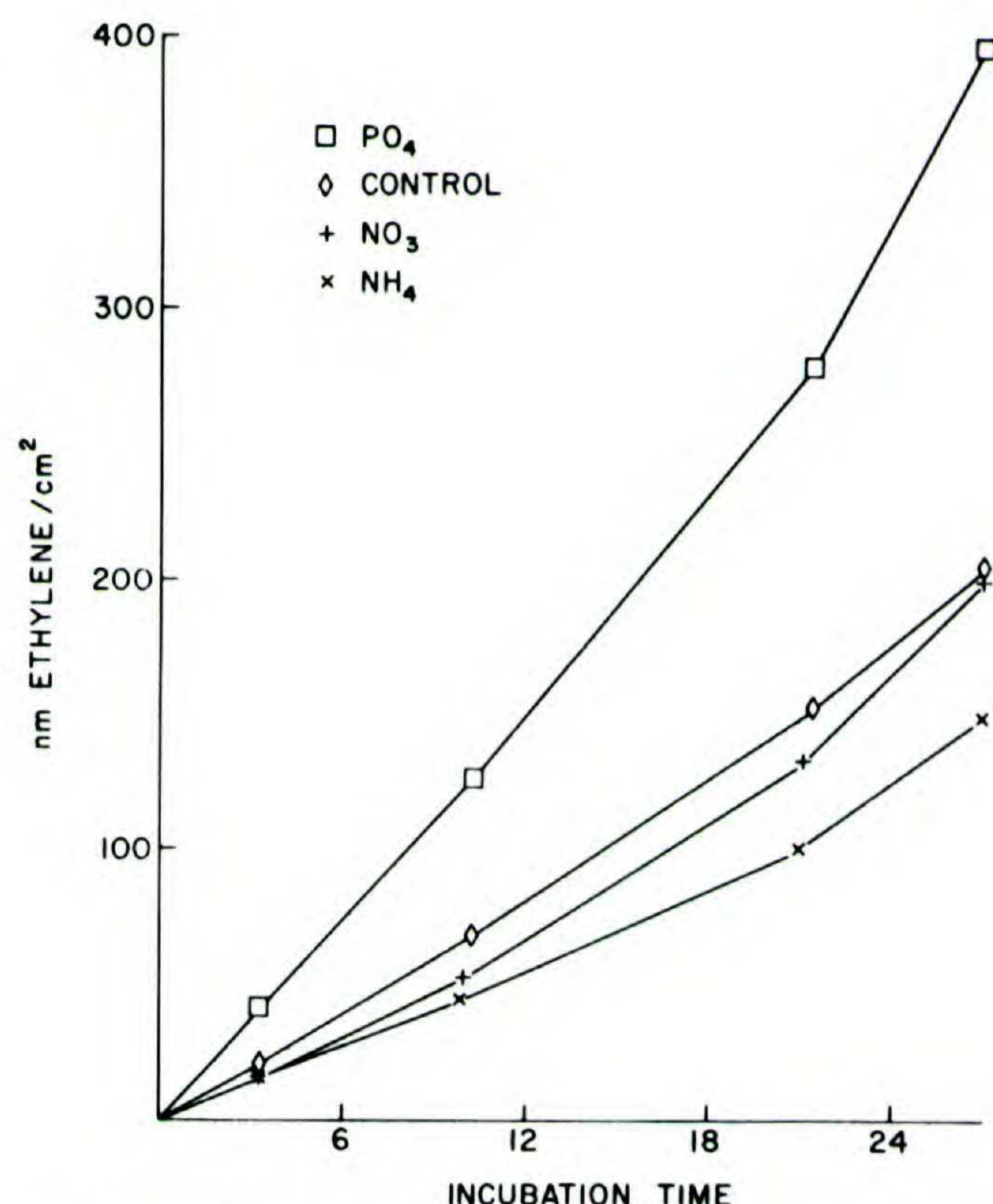


FIGURE 10. Effects of nutrient ions on nitrogen fixation by epiphylls. Leaf samples were dipped into solutions containing $176 \mu\text{g}/\text{liter}$ phosphate as NaH_2PO_4 (\square), $429 \mu\text{g}/\text{liter}$ nitrate as NaNO_3 (+), $125 \mu\text{g}/\text{liter}$ ammonium as NH_4Cl (\times), or deionized water (\diamond). Fixation rates are plotted as nM ethylene to document that fixation continued at a constant rate throughout the 24-hour incubation.

can free an individual plant from competition with its neighbors, which in turn could allow changes in community interactions. In other words, it is the variance in the system which has made this study so interesting.

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